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(54) Title: AMPLIFIED SEQUENCE POLYMORPHISMS (ASPs)

(57) Abstract

A process for the creation of new genetic markers and the simultaneous determination of multiple genetic markers comprising amplifying a genomic DNA sample spanning a sequence variation, detecting DNA sequence variation within the amplified fragment, and detecting the amplified fragments.

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AMPLIFIED SEQUENCE POLYMORPHISMS (ASPs)

This application is a continuation-in-part of application Serial No. 187,428 filed April 28, 1988 entitled "Gene Mapping and Genotyping With PCR Polymorphisms" and incorporated herein by reference.

BACKGROUND OF THE INVENTION

The current initiative to map and sequence the entire human genome portends a detailed molecular understanding of all human genetic diseases. This initiative is derived in part from the use of restriction fragment length polymorphisms (RFLPs) as genetic markers for mapping the human genome.1/ RFLPs are found by screening cloned DNA sequences for their ability to reveal sequence polymorphisms. Restriction enzyme digestion of genomic DNA creates fragment lengths which differ according to the presence or absence of a given restriction site. The fragments are separated by length on a gel, blotted, and hybridized with labeled cloned DNA to display the fragment length polymorphism for homologous fragments. Multiple consecutive hybridizations of such probes to "reusable" Southern blots containing the DNA from appropriate pedigrees are required to map disease loci.

A number of strategies to increase the productivity of gene mapping and genotyping with RFLPs have been developed.

1/ Botstein, D., et al., Construction of a genetic linkage map in man using restriction fragment length polymorphisms, Amer. J. Hum. Genet. 32:314-331 (1980).

Somatic cell hybrids and sorted chromosomes have been used to isolate markers on specific chromosomes.2/ The MspI and TaqI restriction enzymes reveal RFLPs with a higher frequency than other restriction enzymes because their recognition sequence contains the highly mutable CpG dimer.3/ Highly polymorphic RFLPs are found in restriction fragments which span tandem repetitive sequences, such as those associated with the insulin gene4/ and the H-ras gene.5/ Jeffreys et al.6/ discovered a series of polymorphisms by screening with a sequence homologous to many tandem repeat sequences.

2/ Gusella, J.F., et al., Isolation and localization of DNA segments from specific human chromosomes, Proc. Natl. Acad. Sci., USA 77:2829-2833 (1980); Davies, K.E., et al., Cloning of a representative genomic library of the human X chromosome after sorting by flow cytometry, Nature (London) 293:374-376 (1981).

3/ Barker, D., et al., Restriction sites containing CpG show a higher frequency of polymorphisms in human DNA, Cell 36:131-138 (1984).

4/ Bell, G.I., et al., The highly polymorphic region near the human insulin gene is composed of simple tandemly repeating sequences, Nature (London) 295:31-35 (1982).

5/ Capon, D.J., et al., Complete nucleotide sequences of the T24 human bladder carcinoma oncogene and its normal homologue, Nature (London) 302:33-37 (1983).

6/ Jeffreys, A.J., et al, Hypervariable "minisatellite" regions in human DNA, Nature (London) 314:67-73 (1985); Jeffreys, A.J., et al., Individual-specific "fingerprints" of human DNA, Nature (London) 316:76-79 (1985).

Oligonucleotide hybridization is an important technology in the analysis of sequence differences.7/ Nakamura et al.8/ combined the Jeffreys and Wallace technologies to create an efficient strategy for isolating a large number of markers whose polymorphism is based on a variable number of tandem repeats (VNTRs).

Despite this significant progress and the creation of the lymphoblastoid cell bank of the CEPH reference families for gene mapping,9/ much work remains for all of the markers to be well mapped with respect to one another and for the more than 3000 genetic diseases10/ to be mapped with respect to the markers. A more efficient

7/ Wallace, R.B. et al., Hybridization of synthetic oligodeoxyribonucleotides to ϕX174 DNA: The effect of single base pair mismatch, Nucleic Acids Res. 6:3543-3557 (1979); Conner, B.J., et al., Detection of sickle cell β^S-globin allele by hybridization with synthetic oligonucleotides, Proc. Natl. Acad. Sci. USA 80:278-282 (1983).

8/ Nakamura, Y., et al., Variable number tandem repeat (VNTR) markers for human gene mapping, Science 235:1616-1622 (1987).

9/ Dausset, J., Le Centre d'etude du polymorphisme humain, Presse Med. 15:1801-1802 (1986).

10/ McKusick, V.A., Mendelian Inheritance in Man: Catalogs of Autosomal Dominant, Autosomal Recessive, and X-Linked Phenotypes, 7th ed. John Hopkins Univ. Press, Baltimore (1986).

means of mapping new disease and marker loci is needed. This mapping will lead to the disease causing gene. Once the defective gene is isolated, a number of abnormal genotypes are expected to be found. This invention comprises a systematic approach to gene mapping or genotyping based on the analysis, which may be simultaneous, of amplified sequence polymorphisms (ASPs).

Recently, Saiki, Mullis, and collaborators have demonstrated the specific amplification of short genomic DNA sequences by incubating DNA, DNA polymerase, and flanking oligonucleotide primers complementary to opposite strands of the DNA followed by sequential rounds of DNA denaturation and polymerization.11/ The efficiency of each round of polymerization approaches 100% producing an amplification after n rounds of nearly 2^n. This technique, known as polymerase chain reaction (PCR), has been used to detect the sickle cell mutation, 12/

11/ Saiki, R.D., et al., Enzymatic amplification of β-globin genomic sequences and restriction site analysis for diagnosis of sickle cell anemia, Science 230:1350-1354 (1985); Mullis, K., et al., Specific enzymatic amplification of DNA in vitro: The polymerase chain reaction, Cold Spring Harbor Symp. Quant. Biol. 51:263-273 (1986).

12/ Saiki, R.D., supra, n. 11; Chehab, F.F., et al., Detection of sickle cell anemia and thalassaemias, Nature (London) 329:293-294 (1987); Embury, S.H., et al., Rapid prenatal diagnosis of sickle cell anemia by a new method of DNA analysis, N. Engl. J. Med. 316:656-661 (1987).

β-thalassemia mutations,13/ and H-ras mutations.14/ Higuchi, et al.15/ have shown the feasibility of using PCR to amplify DNA obtained from a single hair to detect polymorphisms in mitochondrial DNA and HLA class II genes. These studies have focused on the ability of PCR to amplify the amount of DNA in the sample.

Allele specific PCR (AS-PCR) is described in Wallace, et al. application Serial No. 283,142 filed December 12, 1988 and incorporated herein by reference.

Another enzymatic amplification strategy using DNA ligase is described in Wallace application Serial No. 178,377 filed April 6, 1988 and incorporated herein by reference.

In the ligation amplification reaction (LAR), two pairs of oligonucleotides are ligated. One pair is complementary to adjacent nucleotides on the upper strand and the other pair is complementary to adjacent

13/ Wong, C., et al., Characterization of β-thalassaemia mutations using direct genomic sequencing of amplified single copy DNA, Nature (London) 330:384-386 (1987).

14/ Farr, C.J., et al., Analysis of RAS gene mutations in acute myeloid leukemia by polymerase chain reaction and oligonucleotide probes, Proc. Natl. Acad. Sci. USA 85:1629-1633 (1988).

15/ Higuchi, R., et al., DNA typing from single hairs, Nature (London) 332:543-546 (1988).

nucleotides of the lower strand of the target DNA. The amplification product accumulates exponentially because the products of each round of ligation serve as templates for subsequent rounds. The LAR is favored by perfect base pairing at the ligation site. Under appropriate conditions in the presence of sequence variation, the reaction proceeds for one allele but not for others.

Definitions

For the purposes of this application, the following definitions apply.

Polynucleotide--A nucleic acid molecule composed of more than one nucleotide, e.g., RNA or DNA.

Amplified Sequence Polymorphism (ASP)--The product of amplification as by the PCR or the LAR of a polymorphic target polynucleotide sequence having a preselected locus specific characteristic (LSC), e.g., restriction fragment length, allele specific hybridization capability, gel mobility or a colorimetric or isotopic label.

Marker--The preselected LSC of an ASP.

Locus Specific Fragment (LSF)--A fragment of an amplified polynucleotide having or including an LSC.

Class I ASP--An ASP in which the LSC is the presence or absence of a restriction endonuclease site.

Class II ASP--An ASP in which LSC is a preselected nucleotide deletion or insertion variation in a DNA target sequence or a restriction fragment length polymorphism based on the presence of VNTRs.

Class III ASP--An ASP in which the LSC is a base pair substitution or a small consistent nucleotide deletion not creating an RFLP.

Summary of the Invention

ASP technology provides amplified polynucleotide target sequences containing the variation of a polymorphic locus. The amplified target sequences may be distinguished inter se by a unique physical property, thus facilitating simultaneous, automatic analysis for multiple loci. A preferred physical property is the length or number of nucleotides in the entire amplified target sequence producing an LSF.

The spacing between DNA sequences on a gel is a function of sequence size. ASP technology permits preselection of sequence size with the result that such gel spacing can be maintained substantially constant. In addition, preselected sequence size differences can be resolved on the basis of electrophoresis time over a fixed distance rather than by location on a gel.

The length of amplified target sequences or of LSFs is determined by choice and position of primers used for PCR or for allele specific PCR (AS-PCR). The presence or absence of alleles at each locus may be determined by allele specific primer extension (AS-PE) using primers of predetermined length. The length of an AS-PE product is characteristic of the locus, and the presence or absence of such product is characteristic of the allele of the locus. Preferably, multiple target sequences are simultaneously amplified, and the amplification products simultaneously analyzed.

The invention also includes means for predetermining appropriate PCR primer lengths and a rapid and simple method for preparing DNA for ASP analysis.

Target Sequence Amplification Primers

PCR and LAR amplification primers may be selected or developed either from published information or by sequencing the DNA segments flanking a particular polymorphism.

This aspect of the invention importantly involves the determination of a temperature at which a primer of a given length will prime for a template base of any base composition. This determination is based upon a relationship between the stability of the duplex of an oligonucleotide primer and a template and the

oligonucleotide's ability to prime in a PCR at a given polymerization temperature. This consideration is important because the temperature of polymerization and annealing when PCR is done with a thermostable enzyme, e.g., Thermus aquaticus (Taq), may exceed the temperature at which the primer is stably hybridized to the template.

It is reported that in a primer-template duplex the stability contributed by a G:C base pair is twice that contributed by an A:T base pair.16/ On that assumption, a normalized length, L_n, can be determined by the equation L_n=(number of A and T) + 2 (number of G and C). Based on a systematic study of the temperature at which various primer-template combinations yield a successful PCR product as well as temperatures at which no amplification is observed, a priming temperature at which a primer with a certain length and base composition can be determined. For example, Figure 1 is a curve showing the maximum temperature at which a given primer yielded an amplification product as a function of the normalized

16/ Suggs, et al. in "Developmental Biology using Purified Genes, ICN-UCLA Symposia on Molecular and Cellular Biology," D.D. Brown and C.F. Fox (eds.). Use of synthetic oligonucleotides for the isolation of specific cloned DNA sequences. Academic Press, New York, Vol. 23, No. 54, pp. 683-693 (1981).

length (O) as well as a curve showing the minimum temperature at which no amplification was seen for a given primer as a function of normalized length (•).

The Figure 1 curves reflect data utilizing the primer-template sets set forth in Table I.

TABLE I

RELATIONSHIP BETWEEN PRIMER LENGTH AND SEQUENCE AND ITS ABILITY TO PRIME IN PCR

Gene	Primer Name	Sequence	G+C/L	L_n	T
β-globin	BGP-1	GGGCTGGGCATAAAAGTCA	10/19		
	BGP-2	AATAGACCAATAGGCAGAG	8/19	27	6
H-Ras	H-Ras 5′	CTGTAGGAGGACCCCGGG	13/18		
	H-Ras 3′	CTCTCATGCCCCTCATGCC	12/19	31	6
β-globin	BGP-1	GGGCTGGGCATAAAAGTCA	10/19		
	ON14A	CACCTGACTCCTGA	8/14	22	5
HLA DQα	HLA I	GAAGACATTGTGGCTGACCA	10/20	30	6
	HLA F	ATTGGTAGCAGCGGTAGAGTT	10/21		
HLA DQβ	DQα3 5′	ATGGTCCCTCTGGG	9/14		
	DQα3 3′	GAGCGTTTAATCAC	6/14	20	5
33.6	33.6 5′	TGTGAGTAGAGGAGACCTCA	10/20		
	33.6 3′	AACGTCTGGACAGACAAAGA	9/20	29	6
Insulin	INS 5′	TAAGGCAGGGTGGGAACTAG	11/20		
	INS 3′	GCCACTTTCCACATTAGACC	10/20	30	6
33.4	33.4 5′	ATGGGGGACCGGGCCAGACC	15/20		
	33.4 3′	CCAGGAGGCCACCAGAACCT	13/20	33	7

T_p is the maximum temperature at which priming is observed.
T_n is the minimum temperature at which priming is not observed.
G+C is the number of Gs and Cs in the sequence, and L is the length

Formula to calculate normalized length:

$$L_n=(\#G+C)+(\#A+T)$$

Formula to calculate Tp from the fit of the data:

$$T_p=22.7+1.4(L_n)$$

AS-PCR

AS-PCR provides direct determination of genotype by the presence or absence of a specific amplified sequence. Two allele specific oligonucleotide primers, one specific for a variant allele and one specific for a normal allele, together with another primer complementary to both alleles, are used in the PCR with genomic DNA templates. The allele specific primers differ from each other, for example, in their terminal 3' nucleotide. Under appropriate primer annealing temperature and PCR conditions, these primers only direct amplification on their complementary allele.

An important aspect of this invention therefore is the use of AS-PCR to produce ASPs because a single step yields not only a locus but also an LSF. Simultaneous analysis of multiple loci is facilitated. For example, with 10 sets of primers, 10 individual PCRs may be conducted or the 10 PCRs may be conducted simultaneously. The size of the 10 locus specific sequences in the amplification product may be preset in the range of 100 to 500 base pairs. If the length (L) of the LSF for the ith locus is related to the length of locus i+1 as follows

$\log (L_{(i+1)}) = \log Li + 0.078$ (for 10 loci 100-500 bp)

then the LSAs will be equidistant from each other when resolved on a gel.

Figure 2 illustrates a hypothetical result. P1 and P2 referenced in Figure 2 are the primer sets specific for allele 1 and allele 2 of the particular polymorphic locus. Ten loci each with a different length between 100 and 500 base pairs are shown. The hypothetical individual is heterozygous for loci 1, 3, 6, 8 and 10.

AS-PE

Another important aspect of the invention involves allele specific primer extension (AS-PE) to detect allele variation in PCR amplified target sequences. A DNA polymerase, e.g. Taq polymerase, is used in a primer extension reaction to test for the presence or absence of a particular nucleotide involved in a polymorphism. The following description elucidates this aspect of the invention utilizing sickle cell anemia as a model for single nucleotide polymorphism.

As shown in Figure 3, ASP-A is a 19-nucleotide long primer that is complementary to the antisense strand of the β-globin gene. The primer anneals to the β-globin gene sequence immediately 3′ and adjacent to the nucleotide involved in the A->T transversion mutation of the sickle cell allele. In a primer extension reaction in which only a single α-[^{32}P]dNTP is added, the β^A allele will direct the incorporation of dATP and the β^S allele

1 not 4, regular not terminator (Bajaj)

will direct the incorporation of dTTP. Thus, the primer extension reaction gives rise to a labeled oligonucleotide one nucleotide longer than the primer and in an allele specific way.

The length of the oligonucleotide used in the primer extension reaction can be varied, either by making it complementary to more nucleotides of the template or, preferably, by maintaining the extent of template complementarity and adding additional, non-template complementary nucleotides to the 5' end of the primer. In the latter case, multiple AS-PE reactions can be analyzed on a single gel. Multiple, independent AS-PE extensions can be accomplished in a single reaction.

weight as label?

In a typical AS-PE experiment, 3 μl of a 25 cycle, PCR amplified β-globin DNA sample that has been gel purified to remove the PCR primers was used as the template for the single nucleotide extension of the ASP-A primer. AS-PE reactions were performed with 0.5 μM ASP-A in the presence of 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 1.5 mM $MgCl_2$, 0.01% (w/v) gelatin, 2.5 units of Taq polymerase, and either 3 μM of [α-^{32}P]dATP or [α-^{32}P]TTP (300 Ci/mmol). 10 μl of mineral oil was layered on top of the reaction mixture (10 μl total volume) to prevent evaporation. The sample was then heated for 5 min at 95°C and allowed to incubate at

69°C for 2 hr. At the end of the incubation, 3 μl was removed from the reaction mixture and subjected to electrophoresis on a 7 M urea 20% polyacrylamide gel. The results are shown in Figure 4. Note the absence of a band in lane b and lane c.

In the preferred practice of the invention, a plurality of target sequences are simultaneously amplified. Both non-VNTR loci and VNTR loci may be simultaneously amplified by a single PCR reaction without serious compromise of the amplification efficiency of individual loci.

To test whether three non-VNTR loci could be simultaneously amplified by PCR in a single reaction without an effect on amplification efficiency of individual loci, three well characterized single copy genes, β-globin, Kirsten ras oncogene (K-ras) and growth hormone (hGH), were chosen. PCR was carried out using purified normal human leukocyte DNA as the template. The primer sets used were all 19 or 20 nucleotides in length. Under appropriate conditions (55°C/2 min annealing, 72°C/3 min polymerization using Taq DNA polymerase, and 94°C/1 min denaturation, for 20 cycles), the primer set(s) used single or in combination with the others, each directed the amplification of a single specific major DNA

fragment. The reaction mixture (50 μl) contained in addition to the appropriate buffer, 0.5 μg template DNA, 0.1 mM each of dATP, dCTP, dGTP, and TTP, 12.5 pmol of each primer and 2.5 units of Taq DNA polymerase (Cetus). These amplified DNA fragment(s) were recognizable by ethidium bromide staining following agarose gel electrophoresis and did not require any hybridization step. Under appropriate conditions and using suitable primer sets, multiple, e.g., six to ten, different DNA fragments may be amplified in one reaction mixture.

A similar experiment was done with three VNTR loci 33.6 (Jeffreys, supra), 33.1 (Jeffreys, supra) and H-ras. Specifically, in a 50 μl reaction, 7.5 units Taq polymerase, 25 pmol of each primer and 20 cycles of PCR with the program 67°C/15 sec., 72°C/6 min, 94°C/1 min. It was possible to detect the alleles of all three loci by visualization with ethidium bromide staining or by blotting and hybridization with a mixture of probes specific for the three VNTR loci.

Class I ASPs

Class I ASPs are generally illustrated by Figure 5. The figure illustrates two oligonucleotide primers (P1 and P2) complementary to opposite strands of DNA flanking restriction site polymorphisms in a DNA target sequence.

Four PCR amplified target sequences 1, 2, 3 and 4 are shown. Only sequences 1 and 2 include the amplified restriction site. The presence or absence of the restriction site in amplification product is revealed by digestion with the appropriate enzyme and determining the size of the restriction fragments in known manner.

Class II ASPs

Class II ASPs are generally illustrated by Figure 6 in which P1 and P2 are oligonucleotide primers as described with reference to Figure 5. The PCR amplification product of two DNA sequences of different length (number of nucleotides) is shown. The length difference reflects the deletion or insertion of one or more nucleotides or the number of tandem repeats of a VNTR. Class II ASPs based on the presence of VNTRs are valuable for association studies between specific alleles at the polymorphism marker locus and a disease such as the analysis of the association between the H-Ras tandem repeat and cancer.

Example 1

Primers complementary to the single copy DNA flanking the VNTR region were chosen for the 33.6 locus (Jeffreys, 1985, supra).

DNA (250 ng) from 4 members of a family (father, mother, and two sons) were amplified in a 50 μl reaction containing 12.5 pmol of each primer, 200 μM dNTPs, 2.5 units Taq polymerase and the appropriate buffer. Reactions were overlaid with oil and subjected to 20 cycles of PCR with the following conditions: 67°C/15 sec, 72°C/6 min, 94°C/1 min. The products of the reaction were then subjected to electrophoresis on a 1.5% agarose gel, stained with ethidium bromide and visualized by photographing under UV light. The DNA fragments were then transferred to a nylon membrane (Genetran) by blotting and then hybridized with a probe specific for the VNTR region. After washing, the filter was then exposed to X-ray film.

Figure 7 is a reproduction of the exposed X-Ray film. As the figure shows, in this highly polymorphic locus, three alleles are seen in this family, the father with alleles 1 and 2 and the mother with allele 3 (homozygous). The sons have inherited the alleles from the parents in a mendelian fashion. The example represents the generation of Class II ASPs for this 33.6 locus.

Example 2

Primers were selected in the manner described in Example 1.

DNA (250 ng) from 14 unrelated individuals was amplified in a 50 μl reaction containing 12.5 pmol of each primer, 200 μm dNTPs, 2.5 units Taq polymerase and the appropriate buffer. Reactions were overlaid with oil and subjected to 20 cycles of PCR with the following conditions: 67°C/15 sec, 72°C/6 min, 94°C/1 min. The products of the reaction were then subjected to electrophoresis on a 1.5% agarose gel, stained with ethidium bromide and visualized by photographing under UV light. The DNA fragments were then transferred to a nylon membrane (Genetran) by blotting and then hybridized with a probe specific for the VNTR region. After washing, the filter was scanned on an Ambis Mark II radioactive scanner. The results are shown by Figure 8. As the figure shows, of these 14 individuals, only one appears to be homozygous for the highly polymorphic locus. This example represents the generation of class II ASPs for this locus.

In like manner, the loci for (i) H-ras VNTR, (ii) insulin gene VNTR, (iii) 33.1 (Jeffreys, supra), (iv) 33.4 (Jeffreys, supra), (v) 3'α-globin VNTR, and (vi) ψζ-globin VNTR have been converted into Class II ASPs.

Class III ASPs

Class III ASP generation is typified by Figure 9 in which P1 and P2 are primer oligonucleotides as described with reference to Figure 5. Four amplified DNA sequences which include 2 alleles are shown. Hybridization with allele specific oligonucleotide (ASO) probes simultaneously reveals the location of the allele including the preselected locus specific characteristic as shown by Figure 9. Figure 9 shows one allele that hybridizes (—) to an oligonucleotide specific for allele 1 and does not hybridize (⌐¬) with the oligonucleotide specific for allele 2 and a second allele that hybridizes in the opposite way.

Class III ASPs can also be visualized by hybridizing a blot containing the fragments with a mixture of oligonucleotides, one specific for one allele of each relevant locus. For example, a labelled oligonucleotide probe specific for allele 1 may be mixed with a ten-fold molar excess of unlabeled probe specific for allele 2. Competition hybridization as described in parent

application Serial No. 187,428 with such a mixed probe assures absolute discrimination between the two alleles.17/ After one allele for each locus has been visualized, the probe can be removed and the blot rehybridized with a probe mixture to the other alleles.

Example 3

Oligonucleotides having the sequences shown in Table II were synthesized and purified as described previously.18/

TABLE II

Oligo	Gene[a]	Sequence (5'->3')
Hβ19A	β^A	CTCCTGAGGAGAAGTCTGC
Hβ19S	β^S	CTCCTGTGGAGAAGTCTGC

a The oligos are perfectly complementary either to the normal human β-globin gene (β^A) or to the sickle cell β-globin gene (β^S).

17/ See Nozari, G., et al., Discrimination among the transcripts of the allelic human β-globin genes β^A, β^S and β^C using oligodeoxynucleotide hybridization probes, Gene 43:23-28 (1986).

18/ Wu and Wallace, The ligation amplification reaction (LAR)-amplification of specific DNA sequences using rounds of template dependent ligation, Genomics, in press (1988).

Normal (β^A, β^A), sickle cell disease (β^S, β^S), and sickle cell trait (β^A, β^S) genomic DNA samples were prepared as described by Wu and Wallace, supra, n. 18. β-thalassemia major DNA was prepared from EBV transformed lymphocytes in culture (GM2267 cells from NIGMS Human Genetic Mutant Cell Repository, Camden N.J.). The cells were derived from a patient with a homozygous deletion of the δ- and β-globin genes. Both plasmid and genomic DNA samples were treated with restriction enzymes (Bam HI and/or Taq I) and Exo III nuclease prior to serving as templates in ligation reactions.

PCR primers, BGP1 and BGP2, are 19 nucleotide long synthetic oligonucleotides which anneal to opposite strands of the β-globin gene at positions 256 nucleotides apart (Chehab et al., 1987, supra). 1 μg of genomic DNA samples are routinely used as template in these reactions. Amplification reactions are performed accordingly with the Polymerase Chain Reaction Kit (Perkin-Elmer-Cetus) including 2.5 unit of Taq DNA polymerase and 2.5 μM of oligonucleotide primers. 30 rounds of DNA amplification gave approximately 5 $X10^5$ fold amplification. A 10 μl aliquot of the reaction mixture was subsequently subjected to electrophoresis on a 1.5% agarose gel at 60 V for 5 hr and transferred to a Genetran nylon membrane using 20 X SSC according to Southern

(Southern, 1975). 10 μl aliquots of the reaction mixture are used as template for LAR.

The Genetran membrane was hybridized to labeled oligonucleotide probes, Hβ19S and Hβ19A, in 5X SSPE (1X SSPE= 10 mM sodium phosphate pH 7.0, 0.18 M NaCl, and 1 mM EDTA), 1% NaDodSO4, 10 μg/ml of Homomix RNA, and 10^6 cpm/ml of labeled oligonucleotide with 10 fold excess unlabeled competitor at 47°C for 2 hr (Nozari et al., 1986, supra). The membrane was first washed in room temperature with 6X SSC three times for 30 min (1X SSC = 150 mM NaCl and 15 mM sodium citrate). A subsequent wash in TMACl solution for 1 hr at 59°C removed all detectable mismatch hybridization. (TMACl = 50 mM Tris, pH 8.0, 3 M tetramethylammonium chloride, 2 mM EDTA, 0.25% SDS). The results are shown in Figure 10.

Class III ASPs of particular interest include the common mutations that cause CpG dimers to mutate into TpG or CpA dimers. Class III ASPs are also especially valuable for cloned genes which have not been shown to have RFLPs. If two oligonucleotides, one that includes the wild-type sequence and one that includes the altered sequence, are synthesized, then all of the three possible genotypes can be analyzed unambiguously. Homozygotes for

the presence of one allele will hybridize with the first oligonucleotide; homozygotes for presence of the second allele will hybridize with the second oligonucleotide; heterozygotes will hybridize with both.

In general, high molecular weight genomic DNA, purified by phenol/chloroform extraction of freshly prepared leukocyte nuclei from newly collected heparinized venous blood, is used as the template for primer initiated amplification of specific target sequences by PCR.

One aspect of the invention involves the discovery that the partially purified DNA (for example, by glass powder binding method)19/ from stored nuclei frozen in buffer or in ethanol at about -20°C works well as a template in PCR, compared to the highly purified DNA from freshly prepared nuclei isolated from the same blood specimen. Partially purified DNA prepared by other methods (e.g. digestion of stored nuclei with 0.4M KOH at 80°C for 10 min followed by neutralization with 2N perchloric acid at 0-4°C)20/ failed to amplify specific sequence(s) initiated by the primer set(s).

19/ Vogelstein, B., et al., Preparation and analytical purification of DNA from agarose, Proc.Natl.Acad.Sci.USA 76:615-619 (1979).

20/ McIntyre, P., et al., A quantitative method for analyzing specific DNA sequences directly from whole cells, Anal.Biochem. 174:209-214 (1988).

Figure 11 shows one aspect of the gene mapping utility of ASP technology. A series of i PCR amplified DNA target sequences is depicted. Each amplified target sequence has the length $x_1 + x_2 + C*M_i$, where x_1 is the distance from the first primer to the marker M, x_2 is a constant distance, C is a spacing constant, and M_i is the marker unique to the ith locus. If a series of loci is being analyzed, the ith amplified target sequence including the Marker M_i is derived from the sequence at the ith locus in one individual. If a series of individuals is being analyzed for the same locus, the P2 primer is different for each individual so that the ith amplified target sequence is derived from the sequences at a specific locus in the ith individual.

For example, in a gene mapping experiment, a series of 80 markers (M_i where i = 1,80) well spaced along the genome are selected. For the genotyping of individuals at one or more disease-causing loci, the 80 markers represent target sequence variations or polymorphisms at the marker loci. Oligonucleotide primers are synthesized and positioned so that each of the markers is included in a sequence of unique, locus specific length. For example, when restriction enzymes are used as with Class I and some Class II ASPs, the uncut amplified target sequence lengths

could be $x_1 + x_2 + C*M_i$, and the cut target sequence lengths x_1 and $x_2 + C*M_i$ the locus specific sequence, where C is the number of bases desired between cut sequences. In the case of Class II ASPs which do not involve restriction site polymorphism and Class III ASPs, the series could be simply $x_2 + C*M_i$.

At least four, and preferably five, automation devices are used in ASP technology. Figure 12 illustrates one combination of these devices useful for the complete automation of genotyping and gene mapping experiments. Referring to Figure 12, the combination includes a computer 1, a sample management system 2, an oligonucleotide synthesis machine 3, an oligonucleotide amplification machine 4, and an amplified DNA target sequence analyzer 5.

So understood, the invention includes a single device or system capable of creating oligonucleotides, running amplification and enzyme reactions, and loading reaction products onto an automated gel eletrophoresis instrument, all controlled through a computer with logic for calculating likelihoods of models from pedigree data,[21]

[21] Elston, R.C. and Stewart, J., A general model for the genetic analysis of pedigree data, Hum. Hered. 21:523-542 (1971); Cannings, C., et al., Probability functions on complex pedigrees, Adv. Appl. Probl. 10:26-91 (1978).

for artificial intelligence capabilities, and for oligonucleotide design and control of gene mapping experiments and for conducting a search for ASPs in a region of interest.

Fragment length differences can be very small and thousands of ASPs could be assayed simultaneously on one gel. Bishop and Skolnick[22/] have shown that under many circumstances, gene mapping experiments can yield a set of likely localizations for a gene with a reasonably small number of observations. For mapping a rare dominant disease to a set of genetic markers which are well spaced along the genome and have two common alleles, fewer than 6,000 data points are needed to obtain an expected LOD score of 3 between the disease locus and the closet genetic marker. Therefore one gel would suffice to indicate a set of the most probable map locations for a gene. A second gel would permit selection of the correct location and simultaneously provide a fine structure map.

22/ Bishop, D.T., and Skolnick, M.H., Genetic Markers and Linkage Analysis. In "Banbury Report 14: Recombinant DNA Applications to Human Diseases" (T. Caskey and R. White, Eds.), pp. 251-259, Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y. (1983) and Bishop, D.T. and Skolnick, M.H: Numerical considerations for linkage studies using polymorphic DNA markers in humans. In: Banbury Report No. 4: Cancer Incidence in Defined Populations (J. Cairns, J.L. Lyon, M. Skolnick, eds.), New York: Cold Spring Harbor Laboratory, pp. 421-433 (1980).

If 10 PCRs were prepared in the same reaction, and 50 reactions were prepared per run of a PCR machine, the machine would then have to be loaded 12 times to produce enough reactions to load the initial gel. If a PCR machine were loaded twice a day, and two PCR machines utilized at a time, then a gene would be localized in one work-week. The experiment would proceed even faster, if the PCR machine were further automated and interfaced with an intelligent controller so that reactions occurred around the clock and were collected until the gel was ready to be loaded.

In addition to straightforward gene mapping experiments, ASPs will be useful in a number of other applications. For example, hospital genotyping individuals for a specific disease will have rapid, inexpensive results. Complex genotyping for a large family can be obtained in a single, rapid experiment. A single run through the PCR reaction machine will give a genotypical profile of an individual at hundreds of loci. Such typing is ideal for paternity testing, forensic medicine, or genotyping an individual for loci determining important susceptibilities. For frequent 2 allele polymorphisms, the genotypes of two individuals selected at random from a population have a probability of less

than 1/2 of being identical. Therefore, with 40 marker loci, the probability of wrongly identifying a random individual through his genotype is less than 1 in a 10^{12}.

A number of gene mapping problems require a high density of markers, e.g., the autozygosity method to map rare recessives,23/ the resolution of genetic heterogeneity by simultaneously mapping to multiple loci,24/ and mapping traits that are due to interactions of multiple loci, rare conditions, or highly complex predispositions with low penetrance. Simultaneous ASPs allow the necessary density of markers to be achieved with a reasonable level of effort, thus making these approaches feasible.

ASP technology opens up new possibilities for the study of recombination. A dense map of markers will allow one to identify crossovers precisely, infer chiasma

23/ Smith, C.A.B., The detection of linkage in human genetics, J. R. Stat. Soc. B 15:153-192 (1953); Lander, E.S. and Botstein, D., Homozygosity mapping: A way to map human recessive traits with the DNA of inbred children, Science, 236:1567-1570 (1987).

24/ Lander, E.S. and Botstein, D., Strategies for studying heterogeneous genetic traits in humans by using a linkage map of restriction fragment length polymorphisms, Proc. Natl. Acad. Sci. USA 83:7353-7357 (1986).

distributions, and utilize the information for an analysis of chiasma-based interference.[25] The ability to analyze ASPs on DNA amplified from a single sperm or diploid cell[26] creates new possibilities in gene mapping, genotyping and the analysis of recombination. If each gamete were treated as a haploid offspring so that literally thousands of meiotic products of a single individual could be studied, the map order of very close markers could be resolved and the study of recombination would become even more specific.

ASP technology will also greatly facilitate agricultural experiments in which a gene found in one strain is inserted into another strain through controlled breeding experiments. Genetic markers are required to select the offspring who retain the gene crossed into the strain, who also retain as much of their original genetic compositions as possible.

25/ Goldgar, D.E., and Fain, P.R., A mixed model of genetic interference, Amer. J. Hum. Genet. 41S:A167 (1987).

26/ Li, H.H., et al., Amplification and analysis of DNA sequences in a single human sperm and diploid cells, Nature 335:414-417 (1988).

WE CLAIM:

1. A method for simultaneously determining the genotype at a plurality of polymorphic loci in a polynucleotide target sequence, each of said loci having a preselected measurable locus specific characteristic which comprises:

amplifying at least one polynucleotide target sequence which may or may not include such loci to provide an amplification product containing a plurality of said target sequences; and

analyzing the amplification product to determine the presence or absence of target sequences which include said preselected locus specific characteristic.

2. A method as defined in claim 1 in which the locus specific characteristic at each loci is (i) a restriction site, (ii) the length of a restriction fragment, (iii) a sequence complementary to an allele-specific oligonucleotide probe, (iv) the length of a repeat unit in a VNTR sequence, or (v) a labelled DNA sequence.

3. A method as defined by claim 1 or claim 2 in which the target sequence is amplified by the polymerase chain reaction.

4. A method as defined by claim 1 or claim 2 in which the target sequence is amplified by the ligation amplification reaction.

5. A method as defined by claim 1, 2 or 3 in which the polynucleotide target sequence is a DNA target sequence.

6. A method as defined by claim 1 in which the polynucleotide target sequence is a DNA target sequence and said sequence is amplified by the polymerase chain reaction.

7. A method for determining the genotype at a plurality of polymorphic loci in a polynucleotide target sequence, which loci may or may not include a preselected locus specific characteristic due to (i) deletion/ insertion variations, (ii) variable number tandem repeats, (iii) base pair substitution, or (iv) small consistent deletions which comprises:

amplifying polynucleotide target sequences which may or may not include said preselected locus specific characteristic to produce a product containing a plurality of amplified target sequences of different length;

the length of amplified target sequences in said amplification product having been predetermined by the positioning of the primers utilized in the amplification process on the target sequence; and

visualizing the differences in the lengths of target sequences in the amplification product.

8. A method as defined by claim 7 in which the target sequence is a DNA target sequence which is amplified by a polymerase chain reaction.

9. A method for simultaneously determining the genotype at a plurality of polymorphic loci in a polynucleotide target sequence, which sequence may or may not include a preselected locus specific characteristic restriction site which comprises:

amplifying a target sequence;

digesting the amplification product with a restriction enzyme effective to cleave said product at such preselected restriction sites, if present; and

analyzing at least some of the cleave fragments.

10. A method as defined by claim 9 in which at least some of the cleavage fragments are analyzed by visualizing the length thereof.

11. A method for determining the genotype of a plurality of polymorphic loci in a DNA target sequence, which sequence may or may not include a preselected genotypical enzyme restriction site which comprises:

amplifying a DNA target sequence spanning the said loci by a polymerase chain reaction to produce an amplification product containing a plurality of DNA target sequences;

digesting the amplification product with a restriction enzyme effective to provide fragments flanking said restriction sites, if present, in the DNA target sequences included in said amplification product;

the length of said fragments having been determined by preselection of the number of nucleotides separating said restriction sites on said target DNA from at least one of the oligonucleotide primers utilized in said polymerase chain reaction; and

visualizing the lengths of said fragments.

12. A method for the creation of new genetic markers and the simultaneous determination of multiple genetic markers in a genome DNA sample, comprising:

amplifying said genomic DNA sample to provide an amplification product containing a plurality of sequences which may or may not include said DNA sequence variation; and

determining the presence or absence of DNA sequence variation in said plurality of amplification product sequences.

13. The method of claim 12 in which the sequence variation in said genomic DNA sample comprises a cleavage site of a restriction enzyme.

14. The method of claim 12 in which the sequence variation in said genomic DNA sample comprises the deletion or insertion of a nucleotide.

15. The method of claim 12 in which the sequence variation in said genomic DNA sample comprises the presence of variable number tandem repeats (VNTRs) in said DNA sample.

16. The method of claim 12 in which the sequence variation in said genomic DNA sample comprises base pair substitution polymorphism.

17. The method of claim 12 in which the sequence variation in said genomic DNA sample comprises small consistent nucleotide deletions.

18. The method of claim 12 in which the sequence variation is detected by digestion of the amplified DNA sample with a restriction enzyme and visualizing the resulting DNA fragments.

19. A method for the simultaneous determination of multiple genetic markers comprising:

amplifying by a polymerase chain reaction a genomic DNA sample spanning a preselected enzyme restriction site;

digesting the amplification product with a restriction enzyme effective to cleave said product at said preselected restriction site;

the length of said fragments produced by said cleavage having been predetermined by the number of nucleotides separating said restriction site of said sample from at least one of the oligonucleotide primers used in said polymerase chain reaction; and

analyzing the cleavage product to determine the presence or absence therein of fragments of said predetermined length.

20. A method as defined by claim 19 in which the presence or absence in said cleavage product of a fragment of predetermined length is determined by gel electrophoresis.

21. A method as defined by claim 19 in which the presence or absence in said cleavage product of a fragment of determined length is determined by the location of said fragment on a gel.

22. A method as defined by claim 19 in which the presence or absence of said cleavage product of a fragment of determined length is determined by the elapsed time required for a fragment to appear at a predetermined location on a gel.

23. A method for determining the presence or absence in a DNA target sequence of at least one locus having a preselected, measurable locus specific characteristic flanked by first and second alleles which comprises:

(i) subjecting said DNA target sequence to a polymerase chain reaction using first and second primer sets

said first set of primers being complementary to both of said first and second alleles,

one of said second set of primers being specific only for a first or second allele unique to said locus,

to produce a PCR product in which the only amplified sequences include said preselected locus specific characteristic typical of said locus, and

(ii) determining the presence or absence in said PCR product of sequences including said preselected locus specific characteristic.

24. A method as defined by claim 23 in which step (ii) is accomplished by simultaneously separating the sequences including said preselected genus specific characteristic by gel electrophoresis.

25. A method as defined by claim 23 or claim 24 in which, in step (i), a plurality of DNA target sequences are simultaneously subjected to a polymerase chain reaction using first and second primer sets designed for the allele specific amplification of each of the loci in said DNA target sequences having a preselected locus specific characteristic.

26. A method as defined by claim 3 in which the amplification product is analyzed by

(i) forming a primer template duplex in which the template is an amplified polynucleotide target sequence which may or may not include said locus specific characteristic and the primer is annealed to said template immediately 3′ and adjacent to the nucleotide involved in said locus specific characteristic;

(ii) subjecting said duplex to a polymerase chain reaction in the presence of a labelled αdNTP in which N is a nucleotide complementary either to the next nucleotide in said target sequence which includes said locus specific characteristic or to the next nucleotide in said target sequence which does not include said locus specific characteristic, thereby producing a labeled locus specific extension product one nucleotide longer than said primer; and

(iii) determining the presence or absence of said locus specific extension product in the polymerase chain reaction product of step (ii).

27. A method as defined by claim 26 in which said locus specific characteristic is an endonuclease restriction site.

28. A method which comprises simultaneously amplifying a template DNA target sequence utilizing a plurality of primer sets so designed and positioned with respect to said template as to provide an amplification product including a plurality of amplified target sequences each of unique length and simultaneously separating said amplified target sequences of different length by gel electrophoresis.

29. A method which comprises simultaneously amplifying by the polymerase chain reaction a DNA target sequence having a plurality of polymorphic loci each of said loci having a preselected, restriction site polymorphism flanked by unique alleles,

utilizing in said simultaneous polymerase chain reaction a plurality of primer sets designed to provide an amplification product having flanking alleles of a length unique for each of said loci,

cleaving the polymerase chain reaction product at said preselected restriction sites to produce a plurality of alleles of a length unique to each of said loci, and

determining the presence or absence of alleles unique to each of said loci by gel electrophoresis.

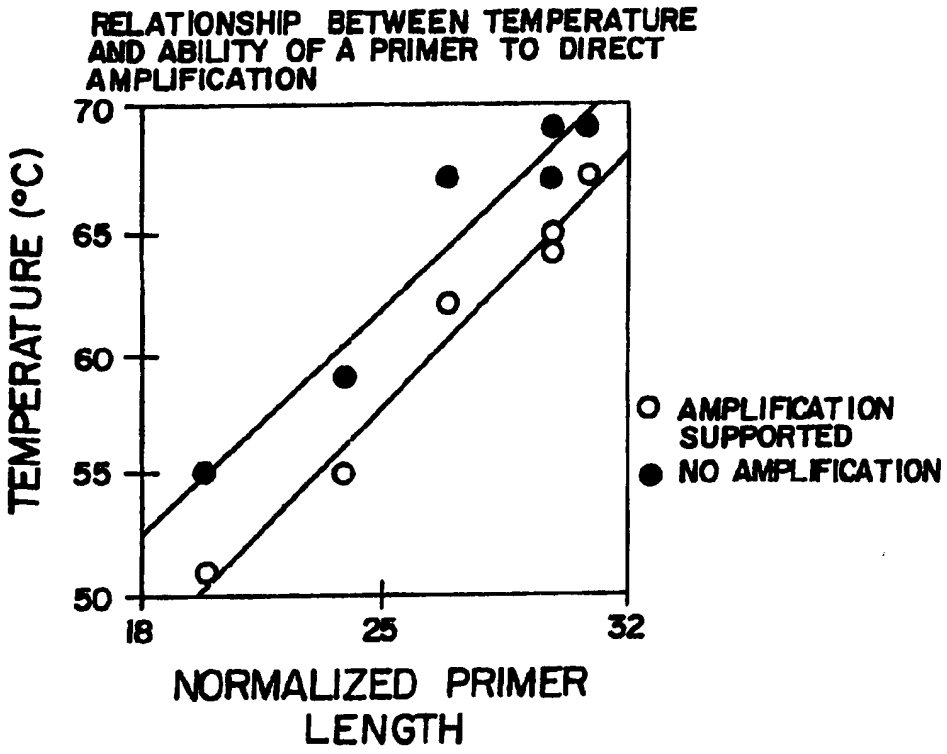
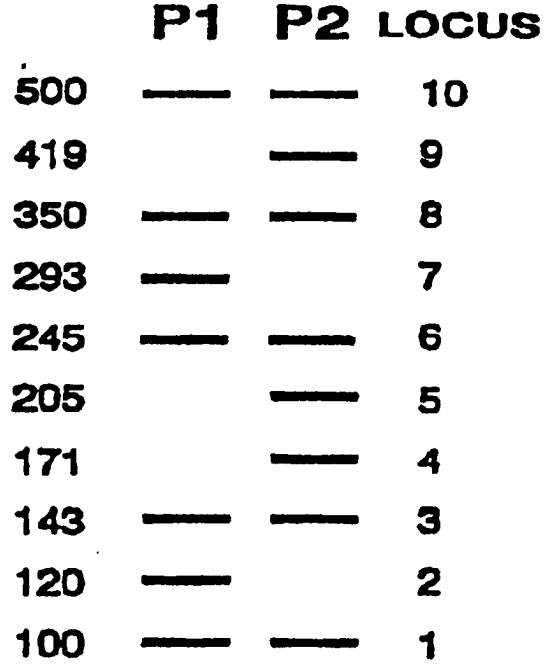
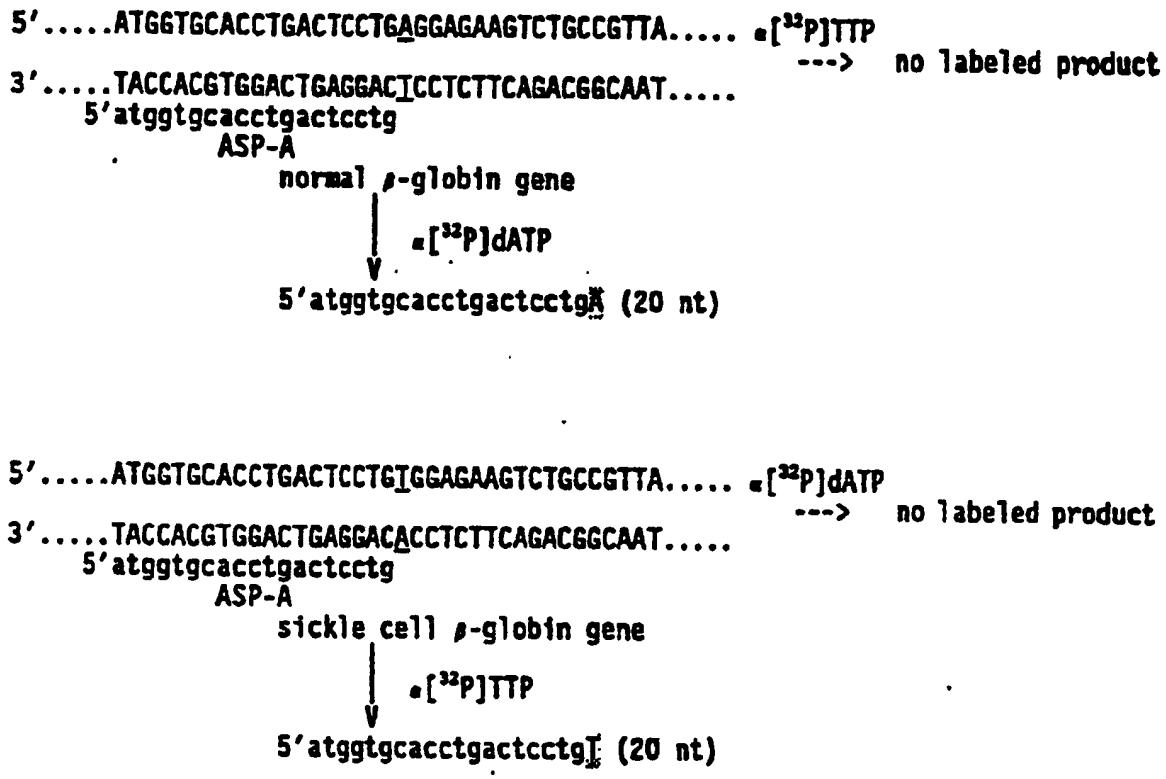

RELATIONSHIP BETWEEN TEMPERATURE AND ABILITY OF A PRIMER TO DIRECT AMPLIFICATION
TEMPERATURE (°C)
70
65
60
55
50
18
25
32
NORMALIZED PRIMER LENGTH
AMPLIFICATION SUPPORTED
NO AMPLIFICATION

FIG. 1



Hypothetical AS-PCR pattern. Individual is heterozygous for loci 1,3,6,8 and 10. P1 and P2 are primer sets specific for allele 1 and allele 2 of the particular locus.

FIG. 2



Oligonucleotide ASP-A is extended with dATP but not TTP on the normal β-globin gene templat and with TTP and not dATP with the sickle cell template. The product is 20 nucleotides long and is labeled when the α-[^{32}P]dNTP is used.

FIG. 3

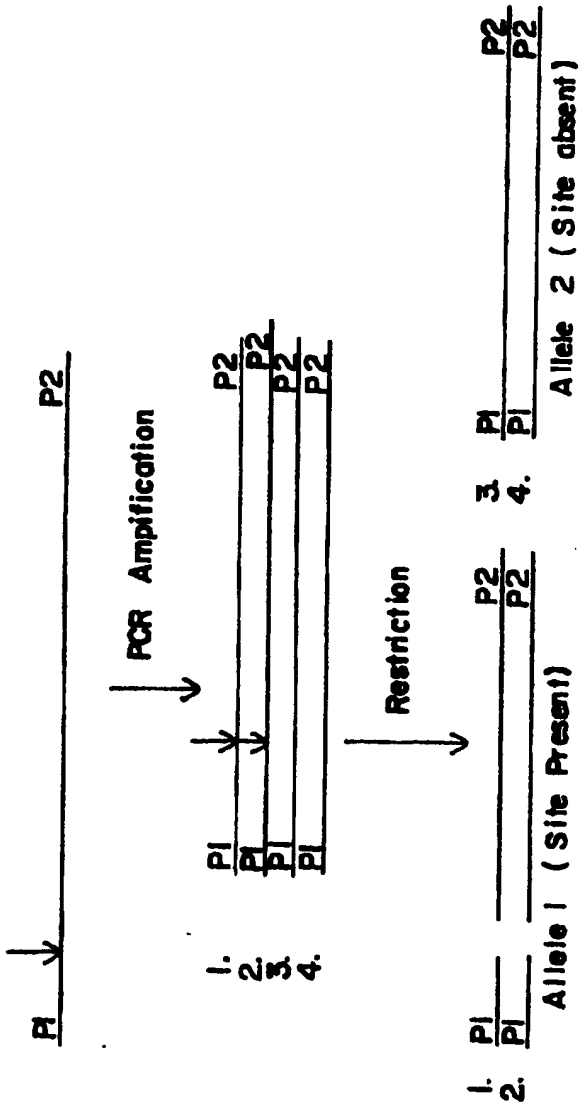

P2
P1
PCR Amplification
1.
2.
3.
4.
Restriction
Allele 1 (Site Present)
Allele 2 (Site absent)

FIG. 5

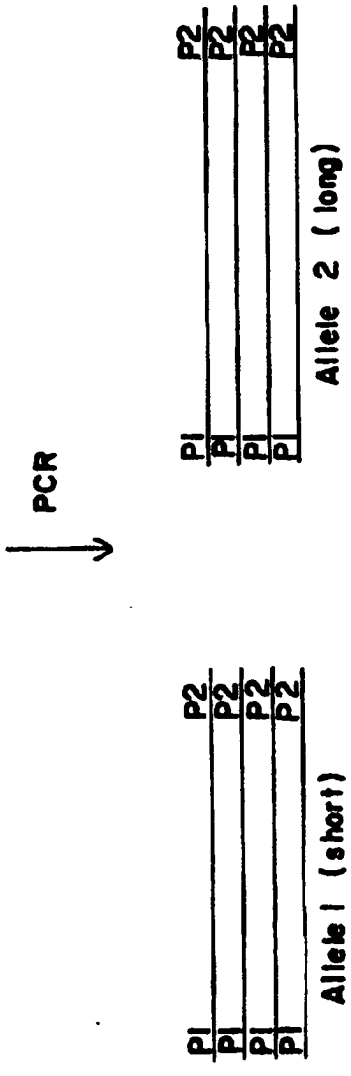

P2
P2
P2
P2
P1
P1
P1
P1
Allele 2 (long)
PCR
P2
P2
P2
P2
P1
P1
P1
P1
Allele 1 (short)

FIG. 6

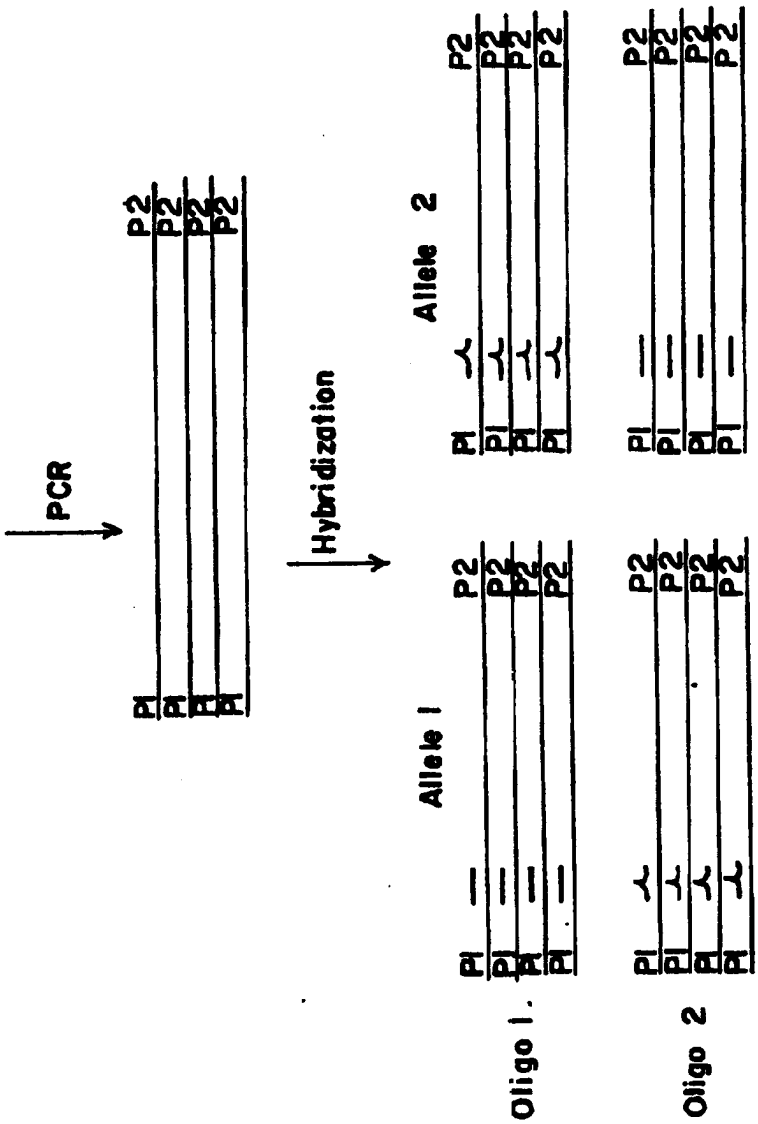


FIG. 9

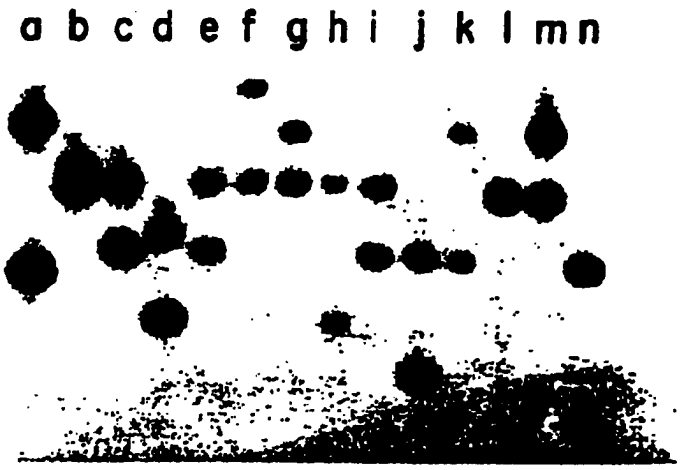
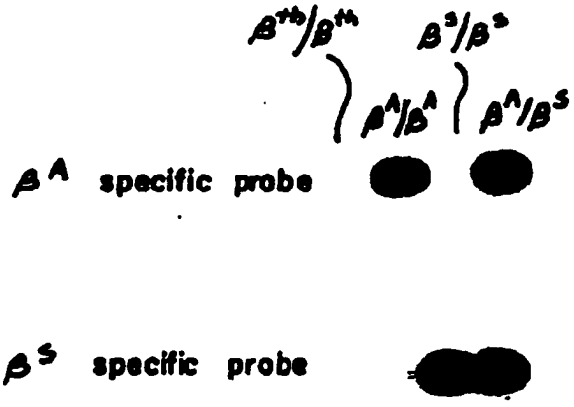


FIG. 8

FIG. 10



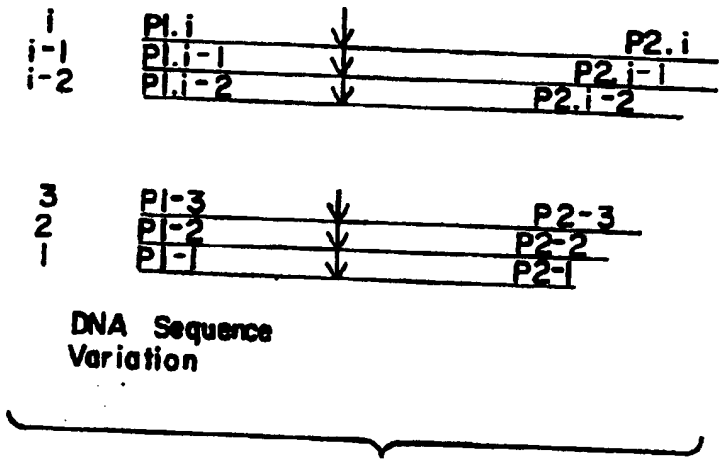

i
i-1
i-2
P1.i
P1.i-1
P1.i-2
P2.i
P2.i-1
P2.i-2
3
2
1
P1-3
P1-2
P1-1
P2-3
P2-2
P2-1
DNA Sequence
Variation

FIG. 11

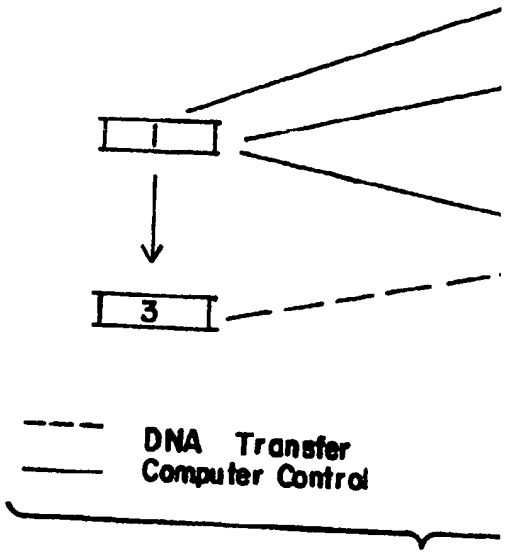
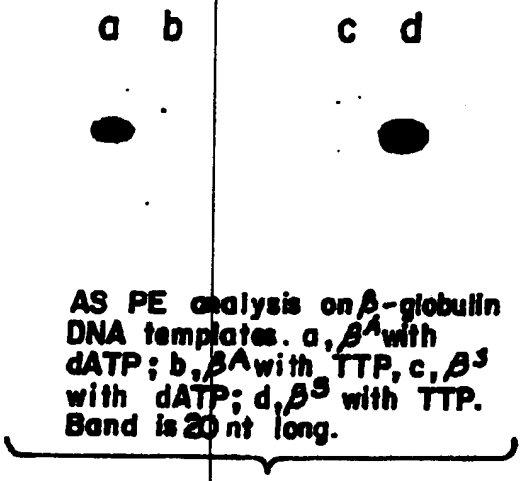

1
3
DNA Transfer
Computer Control

FIG. 12



AS PE analysis on β-globulin DNA templates. a, β^A with dATP; b, β^A with TTP, c, β^S with dATP; d, β^S with TTP. Band is 20 nt long.

FIG. 4

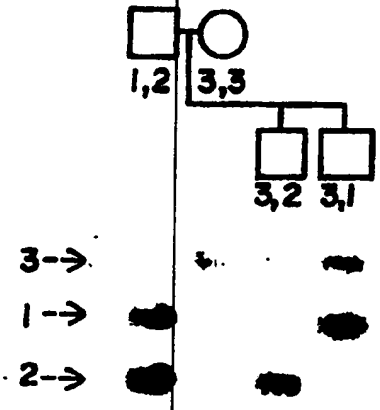


FIG. 7

INTERNATIONAL SEARCH REPORT

International Application No. PCT/US89/01807

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) [6]

According to International Patent Classification (IPC) or to both National Classification and IPC

(IPC) C12Q 1/68; GOIN 30/00
(US) 435/6; 435/91; 536/27

II. FIELDS SEARCHED

Minimum Documentation Searched [7]

Classification System	Classification Symbols
U.S. CL	435/6; 435/91; 536/27

Documentation Searched other than Minimum Documentation to the Extent that such Documents are included in the Fields Searched [8]

APS; CAS; BIOSIS DATABASES

III. DOCUMENTS CONSIDERED TO BE RELEVANT [9]

Category [10]	Citation of Document, [11] with indication, where appropriate, of the relevant passages [12]	Relevant to Claim No. [13]
Y	US, A, 4,683, 195 (Mullis et al) 28 July 1987, see column 18, lines 26-37	1-29
Y	EP A 0,185,494 (Applied Biosystems Inc), 25 June 1986 see abstract	4
Y	Proceedings National Academy Science USA Volume 77, No. 6 issued June 1980 (Washington D.C. USA) Orkin et al. "Cloning and direct examination of a structurally abnormal Beta-thalassemia globin gene" see abstract	1,7-12, 17, 19-28
Y	Cold Spring Harbor Symposia on Quantitative Biology, Volume 51 issued 1986 (Cold Spring Harbor, N.Y., USA) Mullis et al. "Specific Enzymatic Amplification of DNA In Vitro: the Polymerase Chain Reaction" entire article	1-29

* Special categories of cited documents: [10]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
24 July 1989	01 AUG 1989
International Searching Authority	Signature of Authorized Officer
ISA/US	Scott A. Chambers

FURTHER INFORMATION CONTINUED FROM THE SECOND SHEET

X Y	New England Journal of Medicine, Volume 317, No. 16 issued October 1987 (Boston, Mass. USA) Kogan et al. "An improved Method for Prenated Diagnosis of Genetic Diseases by Analysis of Amplified DNA Sequences" see abstract	1-3, 5-29 4
Y	GB, A, 2,135,774A (Actagen Inc) 5 Sep. 1984. see abstract	1-29

V. ☐ OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE [1]

This international search report has not been established in respect of certain claims under Article 17(2) (a) for the following reasons:

1. ☐ Claim numbers . , because they relate to subject matter [12] not required to be searched by this Authority, namely:

2. ☐ Claim numbers . , because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out [13], specifically:

3. ☐ Claim numbers_____, because they are dependent claims not drafted in accordance with the second and third sentences of PCT Rule 6.4(a).

VI. ☐ OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING [2]

This International Searching Authority found multiple inventions in this international application as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims of the international application.

2. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims of the international application for which fees were paid, specifically claims:

3. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim numbers:

4. ☐ As all searchable claims could be searched without effort justifying an additional fee, the International Searching Authority did not invite payment of any additional fee.

Remark on Protest

☐ The additional search fees were accompanied by applicant's protest.

☐ No protest accompanied the payment of additional search fees.

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category *	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No
X Y	Science, Volume 230 issued Dec. 1985 (Washington D.C. USA) Saiki et al. "Enzymatic Amplification Beta-globin Genomic Sequences and Restriction Site Analysis for Diagnosis of Sickle Cell Anemia" see abstract	1-3 4-29